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SEASONAL SEXUAL RHYTHM AND ITS
EXPERIMENTAL MODIFICATION IN
THE MALE OF THE THIRTEEN-
LINED GROUND SQUIRREL
(CITELLUS TRIDECIMLINEATUS)

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1934

By
LEMEN JONATHAN WELLS

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SEASONAL SEXUAL RHYTHM AND ITS EXPERIMENTAL MODIFICATION IN THE MALE OF THE THIRTEEN-LINED GROUND SQUIRREL (*CITELLUS TRIDECEMPLINEATUS*)¹

L. J. WELLS

Hull Zoölogical Laboratory, The University of Chicago

TEN TEXT FIGURES AND ONE PLATE (EIGHT FIGURES)

INTRODUCTION

One of the extremely important problems encountered in sex biology and in clinical pathology is the question of factors which operate to control sexual periodicity. Several investigators in this laboratory have made intensive studies on an annual-breeding rodent (*Citellus tridecemplineatus*), in order to see if the agents governing sexual rhythm could be determined; the comprehensive extent of this work has been outlined in a preliminary paper by Moore, Simmons, Wells, Zalesky and Nelson ('34).

The writer has investigated seasonal variation (under field, laboratory and experimental conditions) in the male reproductive system of this species since July, 1931. Preliminary observations proved this form to be a particularly favorable species for study, since the condition of the genital tract of normal males is such the breeding is impossible for about 10 months of the year; hence, the ground squirrel offers many experimental opportunities, which do not exist in continuous breeders.

This report is a result of a study of the reproductive system from more than 500 males, however, the limited space available in this paper makes it necessary to restrict the number

¹ This study was aided by a grant from the National Research Council, Committee for Sex Research. Grant administered by Prof. F. R. Lillie.

of protocols to a minimum. A more comprehensive presentation of protocols has been given in another paper (Wells, '34).

Some aspects of sexual activity in the male of this species have been studied recently by Johnson and his associates ('31, '33, '34). Relatively recent studies of seasonal variability in male reproductive organs from other mammals have been made (Tandler and Gross, '11; Marshall, '11; Rasmussen, '17; Courrier, '27; Allanson, '32, '33, '34).

The writer wishes to express his sincere gratitude to Dr. Carl R. Moore for stimulating guidance and helpful advice, concerning the investigation reported here, and to Mrs. Rada Wells for presiding as anaesthetist. Many of the experimental studies have been made possible only through the generosity of others, who supplied materials for injection. He is greatly indebted to the following individuals for their courtesy and hearty cooperation: Prof. H. B. Van Dyke and Z. Wallen-Lawrence, for pituitary hebin (an extract of dried sheep pituitary); Prof. F. C. Koch and Dr. T. F. Gallagher, for bull-testis extract; Prof. R. G. Gustavson, for oestrin extracted from human placentae and pregnancy urine; Dr. Oliver Kamm and Parke, Davis & Co., for a pregnancy urine preparation, Antuitrin S; and the staff of the Chicago Lying-in Hospital, Dr. Raymond Kennedy and Dr. Campbell Carey, for urine from women of known periods of pregnancy.

METHODS

Animals were collected alive from the field and brought to the laboratory for autopsy or experimentation. They have been kept available in a laboratory animal room throughout the year, and secured directly from the field from April to November. Only three males were secured by digging out field hibernation burrows; however, many individuals were placed in outdoor terranean-runaways and pens at The University of Chicago and in outdoor pens on a country hillside; they hibernated subterraneously and could be secured from their burrows at any time during the winter hibernation period.

Stock males ran with freedom in a large glass-walled room, located in one corner of a greenhouse. Direct sunlight could enter freely but, since the enclosure was constructed with ordinary window-pane glass, it is probable that very little ultra violet could penetrate. Artificial shade was provided during summer months and a thermostat kept the temperature of the animal room above 20°C. during the winter. The floor of the room was flushed with water and scrubbed weekly. An excessive amount of mixed grain, salt and water was kept accessible to all individuals at all times, while fresh lettuce was supplied daily in excess. Fresh raw meat, whole milk and Purina 'rabbit pellets' were fed frequently.

All experimental animals were housed in wire cages (18 × 12 × 12 inches), which were cleaned weekly and kept in the animal room unless otherwise stated. They were fed in excess, diet and feeding time being the same as for stock males.

Males that had produced spermatozoa at some time were called 'adult,' irrespective of the condition of their reproductive system at the time of study, while those that had never differentiated spermatozoa were called 'young.' Mossman, Lawlah and Bradley ('32) have described the genital tract for eight species of Sciuridae. Their nomenclature for the reproductive organs of *Citellus tridecemlineatus* will be followed in this paper.

All animals were killed with illuminating gas or ether. They were weighed and the following routine autopsy-procedure was used. The degree of scrotal pigmentation and the position of the gonads were determined by observation and palpation. Upon opening the abdominal cavity, an exploratory examination was made for both normal and atypical reproductive organs. Seminal vesicles and one testis were measured, weighed while fresh and fixed for histological preparation. Microscopical study of an epididymal smear for spermatozoon presence and motility, in saline, preceded fixation of the epididymis and the distal vas deferens. Prostate, bulbar and Cowper's glands were measured before being fixed. Post-mortem changes were reduced to a minimum through immediate fixation after death of the animal.

Thin portions of the gross tissues were cut so that relative regions, similar for all males, would be available for microscopic comparison. Tissues were handled by the ordinary paraffin technique after fixation in Bouin's picro-acetic-formol solution; 4 μ sections were stained with Ehrlich's hematoxylin and eosin.

The histological condition of all reproductive organs was studied in much detail and correlated with gross measurements, weights and observations for these structures. Seminiferous-tubule diameter and the epithelial height of all other tissues were measured with an ocular micrometer. The average of five micrometer readings, taken for any one tissue, was recorded in each case.

THE NORMAL SEASONAL SEXUAL CYCLE

The investigation reported in this paper has included the autopsy of approximately 400 male ground squirrels. One hundred and nine animals were sacrificed for the specific purpose of securing all reproductive organs, which were utilized in interpreting the normal seasonal variation in sexual activity. One hundred and twenty-eight of the experimental males contributed normal untreated tissues (testis, epididymis and vas deferens), which were removed surgically before treatment was initiated; although all such tissues were studied in much detail and considered in formulating a conception of sexual periodicity in this species, graphic representation of the seasonal cycle was limited to those animals from which all tissues were available for study (figs. 1 to 10).

Males sacrificed either on the day of capture or shortly thereafter were designated 'field animals,' while those remaining in the greenhouse longer than 1 week were called 'laboratory animals.' These two types of males must be considered separately, since individuals in the laboratory failed to hibernate and reached a peak in the sexual cycle earlier than those from the field. The reproductive cycle will be described, in detail, for field males.

Body weight cannot be utilized as a criterion for distinguishing immature animals from adults, after the young have become 4 or 5 months old. Reproductive glands, however,

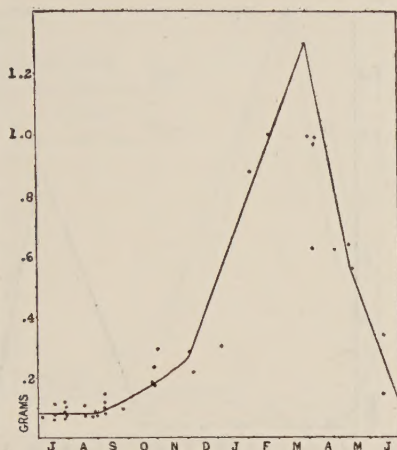


Fig. 1 Seasonal variation in weight of testis from thirty-eight field adults.

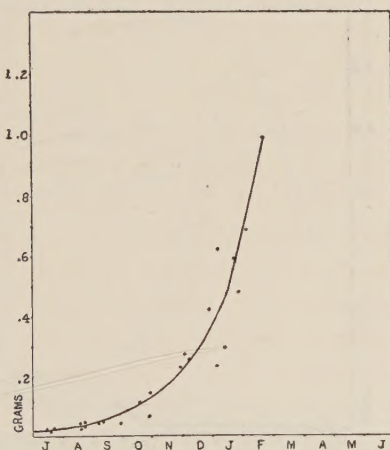


Fig. 2 Seasonal variation in weight of testis from twenty-four young males from the field.

offer a reliable complex of criteria for age determination as late as February, since the genital system of young males develops more slowly than that in adults.

Adult males

Males emerge from hibernation during the first 10 days of April, at which time some of them have the capacity to

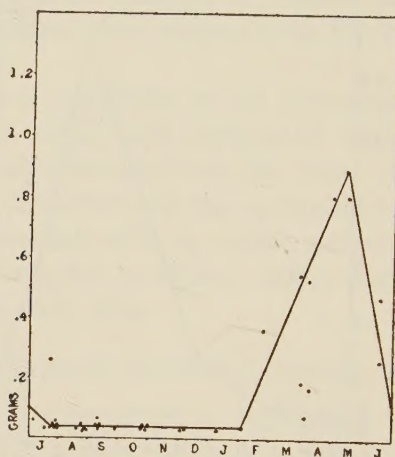


Fig. 3 Seasonal variation in weight of seminal vesicles from thirty-eight field adults.

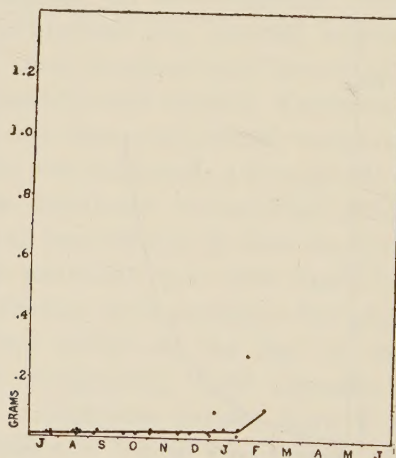


Fig. 4 Seasonal variation in weight of seminal vesicles from twenty-four young males from the field.

copulate while others do not attain this potentiality until a few weeks later. A peak in the function of the reproductive system is reached by most males in either late April or early

May. It is strongly possible that young adults are the last ones to attain maximum sexual development.

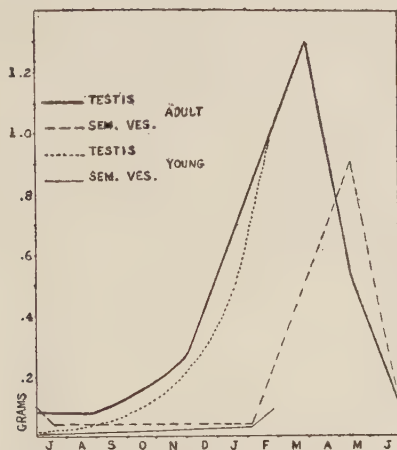


Fig. 5 Superimposition of figures 1, 2, 3 and 4

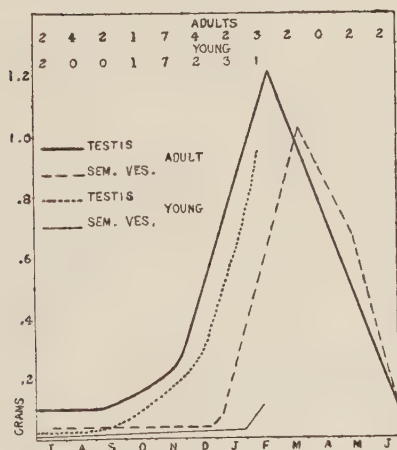


Fig. 6 Seasonal variation in weight of reproductive glands from thirty-one adult and sixteen young laboratory males.

The entire reproductive tract begins to regress by June and becomes completely involuted by either July or August. All field animals that were secured during August exhibited complete genital regression.

A group of seventy-three adult males, which became sexually active later than others collected at the same time (April, 1933), were studied. These animals were inspected

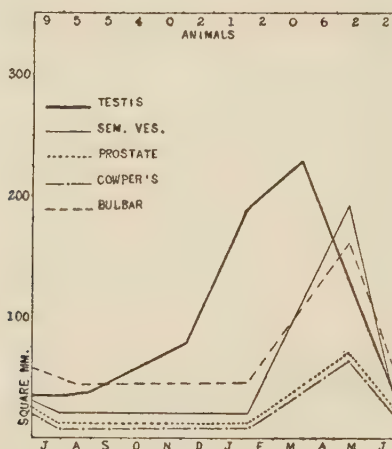


Fig. 7 Seasonal variation in gross size of reproductive glands from thirty-eight field adults.

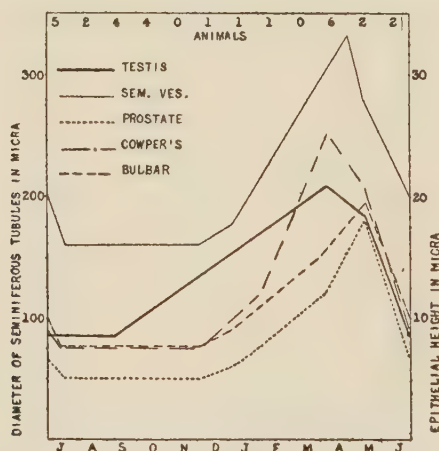


Fig. 8 Seasonal variation in micrometer measurements of reproductive tissues from twenty-eight field adults.

at intervals of 2 weeks during July and August. The condition of reproductive structures was determined by palpation (testis, epididymis and bulbar gland) and observation (scrotal

pigment). The genital system had regressed completely by the first of August in all animals except nine. These nine males showed complete sexual regression either late in August or in September.

The reproductive system is practically inactive between July and December. Renewed activity is initiated during

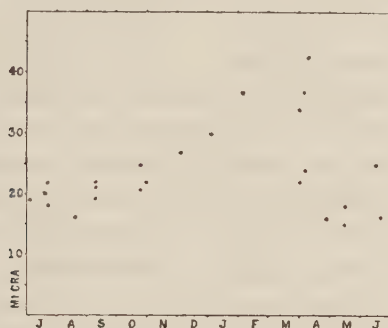


Fig. 9 Seasonal variation in height of tubule epithelium of epididymides from twenty-four field adults.

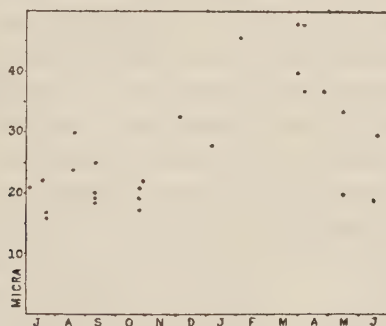


Fig. 10 Seasonal variation in epithelial height of vasa deferentia from twenty-six field adults.

hibernation and progresses gradually, a functional maximum being attained again after emergence in the spring.

Accessory reproductive organs lag behind the testis, both in their augmentation and in their involution. Animals have very large testes and small accessories before the breeding peak is reached and, conversely, they have small testes and functional accessories during early reproductive regression.

Testes are scrotal in position, maximum in size and vigorous in spermatogenetic activity at the time when animals first appear above ground in the spring (figs. 1, 7, 8); all stages of maturation can be found and very many spermatozoa are present during this period. They remain scrotal and active throughout the breeding season, but decrease in size due in part to their release of sperms to the epididymis, since spermatogonia formation does not seem to keep pace with germinal metamorphosis.

The onset of testicular regression, probably depending on the age of the animal, begins sometime between late May and early June. By the middle of June, approximately one-half of the males collected had flabby, abdominal gonads. Such organs are found to be histologically infantile in type; all seminiferous-tubule lumina, secondary spermatocytes, spermatids and spermatozoa have disappeared, while few primary spermatocytes and many spermatogonia remain. Other males collected at the same time had scrotal, turgid and medium-sized testes, which have diminutive seminiferous tubules with small lumina and contain all cellular elements, characteristic of the breeding season, in greatly reduced numbers. All field males that were secured exhibited complete gonadal regression before August 1st.

The small, abdominal gonad retains its flabby condition and spermatogenetic quiescence throughout the summer and just before hibernation begins in October it acquires slight turgidity due, apparently, to an increase in the number of primary spermatocytes. Renewed activity can be detected microscopically before macroscopic changes have become obvious (compare figs. 1, 7, 8). An increased number of primary spermatocytes is accompanied by an increase in testicular size and turgidity, during the early winter hibernation period. Small seminiferous-tubule lumina and secondary spermatocytes appear by the first week in December, while spermatids are formed as the testis reaches the scrotum in January.

During the first week in February, the scrotal gonad is much enlarged and contains large seminiferous tubules with prominent lumina, few spermatogonia, many primary spermatocytes, very many secondary spermatocytes and spermatis and a few spermatozoa. Throughout the remaining period of hibernation, spermatozoa increase progressively in abundance as the testis approaches its weight peak, characteristic of emergent males.

Seminal vesicles (vesiculae seminales) show greatest size, weight and function between the last week of April and the first week in May, i.e., during the breeding season. At this time, the tubules are strutt with transparent secretion and separated from others by very little intertubular connective tissue. The epithelium is cuboidal at this period, since the accumulation of secretion mechanically reduces epithelial height (compare figs. 3, 7, 8).

These glands shrink progressively, both grossly and histologically, after the mating period. All secretion disappears from the tubules, the epithelium becomes extremely low and connective tissue appears in relative abundance by July.

The epithelium remains practically quiescent until January, but thereafter it grows steadily in height and produces secretion during the last portion of the hibernating period; renewal of activity is histologically detectable before gross changes become apparent. As the breeding season approaches, the epithelium becomes high columnar and remains in this condition until the amount of secretion is great enough to exert pressure on it.

The prostate gland (glandula prostatica) differs from the seminal vesicles because it stores no secretion, therefore, its epithelium is highest (low columnar) in breeding males. It exhibits its greatest size and activity during the breeding season; at this time, it is composed of closely packed acini with a decidedly convoluted epithelium, the cytoplasm of which contains few granules and stains lightly in hematoxylin, and very little connective tissue.

This gland regresses both grossly and histologically at the close of the mating period, so that only an occasional acinus with its tiny lumen is present either during late July or in August and connective tissue appears in relative abundance. Epithelium of all other acini has contracted so severely that it is represented by mere clumps of cells, which have lost their epithelial organization.

The prostate remains in this reduced state until February, when it shows an increase in gross size as the acini begin to be organized. The epithelium grows in height, progressively, and becomes convoluted by April 1st.

Cowper's glands (glandulae bulbo-urethrales) undergo seasonal variation similar to that of the seminal vesicles, since secretion is stored in both cases. During the mating period, they are very large and distended with clear viscous secretion, but they contain only traces of connective tissue; the epithelium is low columnar in peripheral tubules and low cuboidal in others, where much of the cytoplasm erodes to produce secretion.

Their involution is simultaneous with that of other accessory organs. Regression is characterized by a loss of epithelial convolutions and a reduction of the epithelium until its cytoplasm is little more than nuclear diameter in height, however, slight traces of secretion and lumina persist in the tubules, which become separated by much connective tissue.

This reduced condition is maintained until January, when histological signs of activity reappear. Early reconstitution is largely restricted to an increase in height of the epithelium and a relative reduction in connective tissue, advance in gross size being noticeable in February and progressing gradually as the breeding period approaches. The epithelium shows its greatest height a few weeks previous to the mating season, since it is reduced mechanically due to the pressure of stored secretion.

The bulbar gland (glandula bulbo-urethralis inferior) behaves somewhat like the prostate gland. Since secretion is not stored, both epithelial height and gross size are greatest

at breeding time. At this period it is composed of a decidedly granular high columnar epithelium, which is very greatly convoluted. Relatively little connective tissue and only a trace of secretion are present.

This gland recedes along with other accessory organs and shows complete involution either in late July or in August, when connective tissue appears in relatively great abundance. Epithelial height is reduced and approximates that of the nucleus, resulting in almost complete obliteration of convolutions and the appearance of a ramifying lumen.

Histological activity is detectable in January while an increase in gross size can be observed in February. The epithelial cytoplasm becomes granular, while the epithelium grows in height and exhibits convolutions during the last part of the hibernating period.

The epididymis (caudal portion) and its tubules are greatest in size during the breeding season. At this time, tubular epithelium and muscle layers are reduced to a point lower than their maximum by the pressure of stored spermatozoa, but cilia are longer than at any other period of the year.

Seasonal regression and relief from spermatozoon distension are simultaneous, so that the progressive character of regression in the epididymis is indicated poorly by micrometer measurements of the epithelium (fig. 9). The epididymis and its tubules decrease decidedly in size and spermatozoa disappear from the lumina by August, at which time muscle layers are thin, the epithelium is low and bears no cilia and connective tissue is relatively prominent.

Renewal of growth begins in early January and continues, even after some spermatozoa have appeared in the tubules in late February. The epithelium is highest and muscle layers are thickest during the first week of April, before the tubules become tremendously expanded by spermatozoon accumulation.

The vas deferens (ductus deferens) exhibits a seasonal cycle, similar to that of the prostate and bulbar glands. Micrometer measurements fail to demonstrate this, however, since the degree of contraction and fixation shrinkage were

not constant for the duct of each animal (fig. 10). During the breeding season it has a large diameter, high columnar epithelium, long cilia and thick muscular coats.

Regression is complete either in July or August, a time when the duct has a reduced diameter, low cuboidal epithelium (lacking cilia) and relatively thin muscular layers. This state persists until January, when cilia buds appear and other components of the tube start on an upward trend, which is continued progressively until the breeding season is reached.

Pigmentation of the scrotal skin shows a seasonal rhythm, similar to that of other secondary sex characters. Scrotal pigment is abundant in breeding males, which have a deep black scrotum. Following the mating season, this pigment gradually diminishes and completely disappears by August. It does not begin to return before January, after which it gradually becomes denser as the breeding period approaches.

In résumé, the entire reproductive system is essentially inactive for a period extending from late July to early December. Spermatogenesis begins during the first part of the hibernation period and progresses to the formation of spermatozoa by February. The testis attains its greatest size approximately 4 weeks before accessory reproductive organs reach a peak in functional activity. The sequence of function within the various organs is significant, since accessory sexual structures (whose function is that of transmitting spermatozoa within an aggregate of semen) exhibit their greatest degree of activity after spermatozoa have escaped from the testis to the epididymis and after the testis has begun to shrink in size.

Young males

Young are born sometime between late May and the first week of June. Somatic growth is so rapid that they are as large as adults by autumn; however, the entire genital system develops slowly during the first 7 months of life. The testis is turgid, even while abdominal, and much smaller than the flabby gonad of sexually regressed adults. Accessory organs grow slowly, previous to the appearance of hormonal stimula-

tion by the testis, and can be distinguished from homologous adult structures, which are larger even while they are in an involuted condition. Accessories of young animals and those of adults are histologically similar; however, the epithelium shows only slight similarity in extremely minute details; it is disrupted or partially disorganized and low in adult structures, but intact (except for the prostate gland, in which a distinct epithelial organization appears relatively late) and slightly higher in young organs. Young males begin to produce spermatozoa from 2 to 3 weeks later than adults and show a similar lag in the development of their accessories (figs. 2, 4, 5, 6).

EXPERIMENTAL STUDIES

From the study of normal animals throughout the year, it was established that the entire reproductive system of adult males is practically quiescent in late summer and early autumn. Several types of experimentation were employed in an attempt to analyze the factors, which operate to produce sexual periodicity in the male of this species.

Bilateral castration

Accessory organs were found to be in an involuted condition in normal adults with abdominal testes. One is confronted with the question, does the testis exert any influence on the accessories during the period of low sexual activity? The experimental approach to an investigation of this question involved the castration of sixteen males, operation being performed either at the breeding season or during the low period. The animals were autopsied following intervals ranging from 4 days to 6 months after castration, during both the mating period and the season of low activity, and their remaining reproductive organs were recovered for study.

In castrates killed during the low season, accessories showed slightly greater histological involution than those secured from normal adults at the same time of the year (compare animals 389 and 739, table 1). No gross effects

TABLE 1

ANIMAL TYPE	NORMAL MALES						
	Untreated		Treated		Untreated		
AGE	Adult		Young	Adult *	Young		
NUMBER	493	739	426	703	348	431	364
DAYS OPERATED					39	26	45
TREATMENT				Cold room			Pregnancy urine
DAILY DOSE							2 cc.
DAYS				160			41
AUTOPSY	4-29-33	11-19-33	11-30-32	4-29-33	11-6-32	1-4-33	11-19-32
Testis 'o'							
S. spermatocytes					0	0	0
Weight—grams					0.0544	0.1296	0.1052
Testis 'a'							
Weight—grams	0.6390	0.2584	0.2207	0.5200	0.3300	0.2617	0.6598
S. spermatocytes	Many	Few	0	Many	Several	Several	Many
Spermatids	Many	0	0	Many	0	0	Many
Spermatozoa	Many	0	0	Many	0	0	Many
Epididymis 'o'							
Epith.—micra					30.0	25.5	27.0
Epididymis 'a'							
Epith.—micra	16.5	24.0		36.0	28.5		22.5
Spermatozoa	Many	0	0	Few	0	0	Many
Vas deferens 'o'							
Epith.—micra					28.5	31.5	
Vas deferens 'a'							
Epith.—micra	37.5	28.5	22.5	36.0	33.0	37.5	49.5
Seminal vesicles							
Weight—grams	0.8100	0.0318	0.0047	0.2354	0.0122	0.0128	1.4690
Epith.—micra	33.5	16.5	12.0	39.0	15.0	10.5	28.5
Prostate							
Size—mm.	9.2×8.0	4.5×2.8	2.3×2.2	5.4×5.1	3.3×2.0	2.3×1.6	9.2×8.2
Epith.—micra	18.0	9.0	7.5	12.0	9.0	9.0	21.0
Cowper's							
Size—mm.	6.3×5.8	3.1×2.5	2.0×1.6	5.0×4.6	2.5×2.1	2.0×1.6	9.0×7.8
Epith.—micra	21.0	7.5	9.0	13.5	10.5	10.5	25.5
Bulbar							
Size—mm.	15.2×7.7	9.9×4.0	6.8×2.5		6.6×2.4	6.6×1.6	20.0×8.0
Epith.—micra	19.5	7.5	9.0	15.0	9.0	9.0	25.5

* Young when treatment was begun. 'o' Removed at operation. 'a' Secured at autopsy.

UNILATERAL CASTRATES						TOTAL CASTRATES		
Treated						Untreated	Treated	
Adult		Young				Adult	Adult	Adult
488	388	352	359	395	363	389	368	369
26	26	34	45	27	32	162	162	162
Oestrin	Male hormone	Male hormone	Pregnancy urine	Antuitrin-S	Pituitary implants		Antuitrin-S	Male hormone
25 R.U.	12 B.U.	6 B.U.	2 cc.	200 R.U.	One		100 R.U.	12 B.U.
26	26	29	41	27	28		29	29
4-29-33	11-26-32	11-6-32	11-19-32	12-10-32	11-5-32	11-6-32	11-6-32	11-6-32
Many 1.1310	Few 0.2826	0 0.0443	0 0.0510	Few 0.1918	0 0.0471			
0.1660 Few Few Few	0.8360 Many Many Many	0.2210 Few 0 0	0.5800 Many Many Many	1.0640 Many Many Many	0.3720 Many Many Several			
30.0	25.5	21.0	21.0	22.5	19.5			
24.0 Few	37.5 Several	49.5 0	25.5 Few	34.5 Few	30.0 0	19.5 0	24.0 0	31.5 0
43.5	30.0	25.5	27.0		19.5			
34.5	36.0	51.0	46.5		31.5	16.5	19.5	
0.1904 18.0	0.6100 31.5	0.4560 31.5	0.9270 30.0	1.5910 25.5	0.1400 18.0	0.0620 15.0	0.0452 10.5	0.9246 27.0
6.6×5.4 10.5	7.8×7.8 19.5	6.5×6.5 13.5	9.8×7.4 19.5	10.0×8.8 22.5	5.0×4.0 15.0	4.0×3.2 4.5	3.6×3.6 4.5	8.9×7.2 18.0
9.4×8.9 10.5	7.7×6.1 24.0	5.9×5.6 21.0	7.8×7.7 27.0	8.5×7.1 27.0	5.0×4.7 22.5	4.0×3.3 7.5	2.8×2.3 7.5	7.5×7.0 22.5
15.1×6.5 52.5	17.0×6.2 25.5	13.6×5.0 18.0	20.0×9.0 21.0	21.4×9.3 34.5	10.6×4.1 19.5	11.3×4.8 7.5	10.0×5.0 6.0	17.5×7.0 24.0

of castration could be detected in accessory organs of castrated males, killed during the low period. Twenty-six days of castration during the breeding season reduced the accessories to an involuted state, similar to that found following normal sexual regression.

It appears, therefore, that the testis may provide slight stimulation for the accessories during the period of low sexual activity. It is felt, however, that a definite answer to this question should await the results of additional castration experiments, now in progress.

Unilateral castration

In all injection experiments (reported later in this paper) one testis, epididymis and vas deferens were surgically removed from each animal, in order to determine the condition of the reproductive system before injections were begun. Possible effects of such unilateral castration were considered, in interpreting the results of injection.

Testis, epididymis and vas deferens were removed unilaterally from nine animals during the period of low genital function and from one during the breeding season. These males received no further treatment but were killed, 16 to 86 days after operation, and their remaining reproductive organs were recovered for gross and microscopical study.

The weight of the remaining testis was decidedly greater than that of the gonad from normal control animals, in all specimens killed 25 or more days after unilateral castration. Spermatogenesis was affected little (if at all) in all periods up to 34 days, but appeared to be accelerated significantly after an interval of 39 days (compare animals 348 and 426, table 1).

Compensatory testicular development was tremendous in two long-time unilateral castrates (73 and 86 days), which were killed during the low season of reproductive activity. Gonad weight was approximately three times that in normal control males and spermatozoa were present, while spermatogenesis had not progressed beyond secondary spermatocytes

in untreated animals. Operation during the breeding season produced no detectable effects on the remaining testis.

Unilateral gonadectomy produced no observable effects on accessory organs and failed to affect spermatogenesis, significantly, in periods up to 39 days. Therefore, it is felt that the remaining reproductive organs of experimental males (discussed later) were altered very little by the surgical removal of tissues preceding treatment, since the injection period was shorter than 39 days in all animals except eight.

Male hormone injection

Normal animals were found to have practically inactive accessories for a period of about 5 months. Male hormone was supplied experimentally, in order to see if such organs have the capacity to function during this interval of time. The hormone (Gallagher-Koch preparation) was extracted either from bull testis or human male urine and dissolved in olive oil. It was administered subcutaneously, during all seasons of the year, recipients being fourteen adult and six young males.

Testis, epididymis and vas deferens were removed unilaterally and injection was begun either on the day of operation or shortly thereafter, except in the case of two 162-day total castrates. Doses of 6 to 15 bird units were administered daily for periods, ranging from 7 to 29 days, until autopsy. All animals were sacrificed between July and November, except two that were killed in April, and all reproductive organs were secured for gross and histological study.

All accessory organs exhibited a decided response in each male, with degree of stimulation depending on period of injection, animal age and the dosage. During the period of low sexual function, 26 to 29 days of treatment produced accessories that were similar to those of normal breeding males; this was true of both unilateral and total castrates (compare animal 388 with 739 and 493; 352 with 348 and 426; 369 with 389 and 493; table 1). Increases in weight of seminal vesicles, induced by male hormone injections ranged between 882 per cent and 10081 per cent.

The testis showed no significant response to male hormone injections, except in each of the four injected males, which were killed during late November. In each of these (one adult and three young), the testis removed at autopsy was scrotal in position and very large in size, and contained many spermatozoa. It should be noted that the time of autopsy (late November) was from 1 to 2 months earlier than the time when spermatozoa appear in uninjected laboratory animals (see animal 431, table 1). The precocious production of spermatozoa by male hormone injections, at the time of year near that for the appearance of normal acceleration in sexual activity, raises the question—is male hormone essential for definitive spermatogenesis? Further discussion of this question awaits a more detailed analysis and will be presented in another paper, to be published in the near future.

Thus it was demonstrated that the accessory reproductive organs have the ability to function at any time of the year, if stimulated by male hormone; the periodic appearance of low activity in accessories of male ground squirrels, therefore, is due to a seasonal deficiency of this substance.

Oestrin administration

The female hormone oestrin (Gustavson preparation) was given to male ground squirrels in order to determine its effect on the reproductive organs. Seventeen animals were injected subcutaneously and autopsied either during the breeding season or during the period of low sexual activity. Removal of one testis, epididymis and vas deferens preceded the first injection, which was applied on the day of operation except in the case of two 162-day bilateral castrates. Daily doses of either 12.5 or 25 rat units were given for intervals varying from 7 to 34 days.

The remaining testis in males killed during the spring mating period appeared either abdominal in position, small, flabby and histologically infantile in type or severely damaged (compare animals 488 and 493, table 1), depending on injection interval and dosage. All accessory organs of such

animals were decidedly atypical, both grossly and histologically; seminal vesicle weight was found to be only about 20 to 25 per cent as great as that for untreated controls. Oestrin treatments inhibited testicular growth, spermatogenesis and normal development of accessories in young animals. Gonadal weight for young injected males was approximately 30 per cent of that for uninjected unilaterally castrated controls.

Unilateral and total castrates, injected with oestrin and sacrificed during the normal low period of sexual activity, revealed a peculiar response on the part of their accessory organs. Such accessory structures were found to have been increased in gross size, by oestrin, when compared with those of uninjected controls. Histological examination, however, showed this to be not a normal secretory response (such as that induced by male hormone) but more particularly an increase in connective tissue and muscle, leucocytosis being conspicuous. The prostate, bulbar and Cowper's glands exhibited a bizarre combination of connective tissue augmentation with epithelial stratification and cornification, atypical for normal males.

It is seen from these results that the entire reproductive system of the male can be injured during the breeding season by oestrin injections. During the period of low genital action, oestrin induced marked changes in accessory organs; however, such organs were atypical for accessories of normal males (at any stage of the seasonal cycle).

Injection of gonad stimulants

The reproductive system of the male is extremely labile at all time of the year, since practically quiescent accessories were stimulated to a functional condition by male hormone additions and the entire genital tract was inhibited during the breeding season by oestrin applications. Gonadotropic substances were administered, in order to see if they would stimulate reproductive organs during the period of low sexual activity.

Untreated urine from pregnant women, between the seventh and eighth month of pregnancy, was given to twenty-six males. Injections were begun from 1 to 4 days after a surgical removal of one testis, epididymis and vas deferens, except in the case of two 205-day bilateral castrates. Each animal received subcutaneous injections (0.5 cc. to 1.0 cc., twice daily) for intervals of 3 to 47 days until autopsy, performed within each month from August to December. All reproductive organs were either measured or weighed and prepared for histological study.

The remaining testis descended into the scrotum within from 5 to 8 days after the initial injection. Its weight (depending on duration of treatment and date of sacrifice) at autopsy was approximately two to ten times greater than that for the gonad of unilaterally castrated and normal controls (compare animals 364 and 739; 359, 348 and 426; table 1). Spermatozoa were formed precociously (compare fig. 14 with 11, 12, 13; 16 with 15), in the treated testis from each of the thirteen males, injected for 27 or more days (27, 30, 31, 33, 34, 40, 41 and 47 days). Motility of spermatozoa from the epididymis of seven such males was observed. With injection periods shorter than 21 days, spermatogenetic responses are questionable; spermatogenesis was accelerated significantly to spermatid formation, however, in each of the four animals receiving 21 to 22 days of treatment.

All accessories showed great gross and histological stimulation in each case, being equal to those of breeding males in eleven animals (both adult and young). Increases in weight of seminal vesicles, stimulated by pregnancy urine injections, ranged from 205 per cent to 11244 per cent.

The response by accessories of bilateral castrates was similar to that induced by oestrin, administered to either unilateral or total castrates. It is probable that oestrin, known to be present in pregnancy urine, was the hormone producing this effect. Gonadectomized animals showed no scrotal pigmentation even after 41 days of treatment, but their secondary reproductive organs were larger than those of uninjected

castrated controls; histologically, these tissues exhibited the atypical oestrin effect. Since no normal stimulation was produced in castrated males, pregnancy urine must act indirectly, through the testis, in stimulating accessories of unilateral castrates.

Antuitrin-S, (Parke, Davis & Co.) was administered subcutaneously to ten males during the period of low sexual activity. One testis, epididymis and vas deferens were removed from each animal, which received its first injection immediately (except in the case of two 162-day total castrates) following operation. Doses ranging from 2.5 to 100 rat units were continued twice daily, for periods from 7 to 29 days, until autopsy. Sacrifice occurred within the interval from July 31st to January 4th; all residual reproductive organs were removed and studied, both macroscopically and histologically.

The remaining gonad of all treated males descended to the scrotum and weighed approximately two to four times more than that of uninjected unilaterally castrated controls (compare animals 395 and 431, table 1). Spermatozoa were induced precociously in each of the seven animals injected for periods from 20 to 29 days, while spermatozoon motility was observed for only one of the injected individuals (compare figs. 17 and 18).

Accessories of each unilateral castrate were stimulated by *Antuitrin-S* and were similar (both grossly and histologically) to those of normal breeding males, in each of the four animals receiving 35 (or more) rat units daily for periods ranging from 26 to 29 days. Accessory organs of the two gonadectomized males exhibited no detectable response to *Antuitrin-S*, when they were compared both grossly and histologically with those of uninjected castrates (compare animals 368 and 389, table 1).

Pituitary hebin (extracted from sheep pituitaries by Van Dyke and Wallen-Lawrence) was given to two young males, during the period of low genital activity. One testis, epididymis and vas deferens were removed and each animal was

injected with 1.5 cc. daily until autopsy. Prolonged injection periods were planned, but the extract induced severe somatic debility, which made it necessary to kill one animal after 14 days of injection (December 30th) and the other after 20 days (January 5th). All remaining genital structures were greatly stimulated in each male, weight of the treated testis being approximately two to four times greater than that of the gonad from uninjected unilaterally castrated and normal controls.

Spermatogenesis was significantly accelerated in each animal; the treated testicle of each animal had several spermatids, while the testis of normal young males and of a 26-day unilaterally castrated control (killed January 4th) contained none. Accessories were stimulated, both grossly and histologically, in each animal. Accessory organs of the male receiving twenty daily injections showed development almost identical with that found in normal breeding adults; seminal vesicles of this male weighed forty-eight times those from the untreated unilateral castrate mentioned above.

Implants of fresh anterior pituitaries from rats were given to one sexually regressed adult and to two young males, during the season of low genital activity. One gonad, epididymis and vas deferens were removed from each animal and the first implantation was made either immediately after operation or after an interval of 3 days. The anterior lobe of fresh rat hypophyses was dissected free and hashed very finely with scissors. This mass was then drawn into a large hypodermic needle, using a minimum amount of normal saline, and injected intramuscularly to the leg of the ground squirrel recipient by means of a syringe. Anterior lobes of two or more glands were hashed together and administered simultaneously, every odd day, to one young male (twenty-two lobes in 16 days) and the adult (twenty-nine in 28 days). The other young male received one lobe daily for 28 days. Implantation continued until autopsy, at which time all remaining reproductive organs were secured for gross and microscopical study.

This treatment stimulated all reproductive structures in each animal. The treated gonad weighed from 1.3 to 4.7 times more than the testicle of unilaterally castrated controls (compare animal 363 with 426, 431 and 348; table 1). Daily implantation of anterior pituitaries, during October and early November, accelerated spermatogenesis to a point of spermatid and spermatozoon formation; neither spermatids nor spermatozoa were found in either normal young animals or untreated unilateral castrates, killed at this time. Spermatogenetic acceleration was induced less effectively (if at all) in the two males receiving implants every odd day; response in each case was limited to a questionably significant increase in the number of primary spermatocytes and the appearance of several secondary spermatocytes, which were not found in controls killed at the same time of year.

All accessories of each animal showed both gross and histological stimulation. All accessory organs of a young male presented a histological picture, which may be designated as approximately 80 per cent of a maximum for normal breeding males, following twenty-eight daily pituitary implants.

The results show that the whole reproductive system of the male can be stimulated to a functional breeding condition, during the period of low sexual activity, by administration of gonadotropic substances. Such materials failed to stimulate accessories of total castrates, but caused the testis of unilateral castrates to release enough male hormone to stimulate development of the accessory organs to the condition of breeding males. Spermatogenetic acceleration, or at least a maturation of existing germinal cells to form spermatozoa, was induced by the following gonadotropic substances: pregnancy urine, Antuitrin-S and fresh pituitary.

*Seasonal sex-stimulating capacity of the ground squirrel
pituitary*

It was found that the whole genital tract of the male is labile and will respond to gonad stimulants at any time. The long annual period of low sexual activity in males of this

species, therefore, is due to a periodic deficiency of testicular stimulant. Anterior pituitary substance proved to be a gonad stimulator for the male ground squirrel, hence, ground squirrel pituitaries were assayed, in order to see if there was a seasonal variation in their sex-stimulating potentialities.

Bio-assays of fresh pituitaries from seventy-two male ground squirrels were made, employing the method of Smith and Engle ('27) with the immature female rat as test animal. Females of a rat litter were selected on the twenty-first day of life, some to serve as pituitary recipients and others as untreated controls.

Six ground squirrels, whose reproductive systems showed approximately the same degree of activity (determined by weights and measurements of testis and seminal vesicles), were chosen to furnish pituitaries for each recipient rat. Anterior lobes from two donors were weighed, hashed very finely and injected intramuscularly in the posterior leg of the recipient. Each treated rat was given two lobes daily for 3 consecutive days, uninjected on the fourth day and killed on the fifth day, when 25 days of age. Experimental rats and littermate controls were killed simultaneously, weighed and examined for rupture of the vaginal membrane. A vaginal smear was studied microscopically in all rats with open vaginae. Weight of the uterus and weight of both ovaries with their oviducts were recorded. Assays were made of anterior pituitaries from: 1) males with scrotal testes; accessories (a) small, (b) medium sized and (c) large; 2) males with abdominal testes, (a) adult and (b) young; 3) castrates, (a) operated prepuberally; tested during the breeding period; (b) operated following normal sexual regression; tested during the season of low sexual activity.

Anterior pituitaries from normal males with scrotal testes induced rupture of the vaginal membrane and the production of a cornified vaginal smear characteristic of oestrus, except in one case where the membrane was practically ruptured and the smear was pro-oestral. Ovarian weight was 2.03 to 3.72 times greater than that of untreated littermate controls,

while uterine weight was 2.86 to 6.58 times greater than that for controls.

Pituitaries from normal males (either young or adult) with abdominal testes produced little or no significant stimulation of either vagina, uterus or ovaries of the recipient young rat; the vaginal membrane failed to rupture in each case. During the latter part of June, sexual organs of some males show only partial regression while those of others exhibit practically complete involution; simultaneous assays showed the pituitaries of males with scrotal testes to have great sex-stimulating capacity and those of males with abdominal testes to have little or none.

Pituitaries from total castrates induced significant hypertrophy of ovaries and uterus, without exception, both during the season of low sexual activity and during the breeding period. All rats receiving castrate pituitaries, except one, exhibited a rupture of the vaginal membrane and an oestral smear; in the exceptional case, the vagina showed a thinned membrane and a pro-oestral smear, while the ovaries and the uterus weighed 3.63 and 5.95 times more than those of controls.

Blood serum was collected from each of the seventy-two pituitary donors and tested for seasonal variation in its sex-stimulating capacity. Immature female rats were given 1 cc. of blood serum, twice daily, for either 3 or 5 days. One day elapsed between the last injection and autopsy, which was performed for both injected females and their untreated sisters. Females of a litter were killed either on the twenty-fifth or twenty-seventh day of life and autopsy procedure was identical with that for pituitary recipients, described above. After the termination of several assays, the 3-day period of treatment appeared to be too short for inducing unquestionable responses, hence, a 5-day injection period was used for all subsequent tests.

Two recipients receiving blood serum from ground squirrels during the breeding season showed marked stimulation of reproductive organs. One of these was given serum from

normal breeding males and exhibited a thinned vaginal membrane (almost ruptured) at autopsy; weight of its uterus was 4.05 times greater than that of an untreated sister, but weight of its ovaries was only 1.47 times greater than that of the control. The other recipient received serum from castrated ground squirrels and showed definite signs of oestrus (ruptured vaginal membrane and cornified vaginal smear) when autopsied; its ovaries and its uterus weighed 4.63 and 4.53 times more than corresponding organs from an untreated littermate.

During the season of low genital activity, no effects of significance were produced by serum from males with abdominal testes, while questionably significant stimulation was induced by serum from gonadectomized ground squirrels. It is futile to attempt a quantitative comparison of the assays made at this time of year (rats injected for 3 days) with those performed during the breeding season (recipients injected for 5 days), since the length of the injection period was not constant for all rats.

These fragmentary data from blood serum assays are interpreted to have no more than suggestive value, but they follow the trend of results from seasonal assays of the potency of the anterior pituitary. These data suggest that the blood stream contains much sex-stimulating material during the breeding season and little or none while the testes are abdominal in position; castration appears to increase the quantity of gonad-stimulating substance in the blood.

Results of these studies demonstrate that the anterior pituitary of male ground squirrels show a seasonal variation in its sex-stimulating potentiality. This capacity is extremely great in animals with scrotal testes and either very minute or absent in males with abdominal gonads. Hypophyses of castrates were found to contain much sex-stimulation material, even during the season of low genital activity (for normal males). Blood serum assays appear to offer suggestive support for these findings. Tabulated data supporting these conclusions are presented elsewhere (Wells, '34).

Experimental changes in the environment

Results of the experiments reported above unite in indicating that the anterior pituitary is the regulating mechanism for reproductive activity in the male ground squirrel, the sexual cycle being due to a seasonal periodicity in pituitary activity. There is still the question, why is the pituitary periodic in its action? Since the male exhibits an annual reproductive cycle, it is certain that some environmental factor operates, possibly either directly or indirectly on the pituitary, to produce sexual periodicity in this species. Males were subjected to various changes in the environment, in order to see if this factor could be determined.

Electric illumination was given to both adult and young males, during the period of low sexual activity, in order to see if a progressive increase in length of the daily light period would stimulate the reproductive system. Daily light additions were designed to simulate the conditions in nature during the spring, when accelerated sexual activity is accompanied by a progressive increase in daylight.

On September 21st when there were 12 hours of daylight, males were distributed to both experimental and control cages, after testis, epididymis and bulbar gland had been palpated. All animals had abdominal testes and non-functional accessories on this day. Eight experimental and four control males were kept in a constant temperature room at 15.5°C., humidity ranging from 70 to 80 per cent. Eight were housed in greenhouse cages, having a temperature of approximately 24°C.

Shaded light bulbs were placed above experimental cages, so as to deliver either 80 or 160 foot candles to the floor of the cages, and lights were turned on at 6.00 A.M. each morning. Electric time-switches turned the lights off, each evening, 15 minutes later than on the preceding day. Length of the light period was increased 15 minutes daily, until animals received 20 hours of light; thereafter, the lights burned 20 hours daily until April 15th.

Light-treated males were autopsied after periods, ranging from 34 to 207 days following the initiation of treatment. All their reproductive organs were compared, both grossly and histologically, with those of control individuals from a diffuse-light cage (0.5 foot candle) and with those of untreated males (from the greenhouse and from the field). No effects, which could be attributed to light manipulation, were detectable; the entire genital apparatus of experimental males appeared similar to that of controls, killed for comparison.

Constant cold temperature was tested experimentally, in order to see if it would stimulate the reproductive system of males, during the period of low genital activity. Experiments were designed to approximate the conditions found within the natural hibernation burrow, where torpidity is associated with low temperature and darkness. On November 20th, three males having abdominal gonads and non-functional accessories were placed within cages in a dark room, having a constant temperature of 4°C., and were examined daily for 128 days. They hibernated periodically, occasionally becoming active and eating.

Testes of each male were palpated frequently and autopsy occurred after periods of 69 to 160 days following the first day of treatment. All reproductive organs were compared, both grossly and histologically, with those from normal laboratory and field males and with those from males, kept in a cool diffuse-light cage (0.5 foot candle; 15.5°C.). Each of the three animals showed significant retardation of sexual development, but the reproductive system appeared normal in all other respects (compare animals 703 and 493, table 1).

Results of these experiments show that the male reproductive system failed to respond to a progressive increase in length of the daily light period and was retarded in its development by constant cold temperature. Cool temperature and practically complete darkness produced no observable effects. Genital organs of two males, dug from hibernating pen burrows in December and kept in the laboratory for 30 days until autopsy, were found to be similar to those from

normal laboratory males. It is evident, therefore, that the environmental factor participating in the control of the anterior pituitary is as yet undetermined.

DISCUSSION AND SUMMARY

Fertility and breeding capacity of the male ground squirrel (*Citellus tridecemlineatus*) is restricted to a short period, during April and May in most field animals. During summer, spermatogenetic activity ceases, gonads decrease in size and recede into the abdomen; all accessory genitalia undergo involution and sterility ensues until the following spring. Non-hibernating laboratory males reach a peak in sexual development earlier than field animals, but retain the characteristic single annual cycle.

Accessory organs at their lowest point of activity appear to have been reduced to a slightly lower level of involution by total castration. Unilateral gonadectomy produced no observable effects on accessories, but resulted in compensatory hypertrophy and accelerated spermatogenesis in the remaining testis, after long post-operative periods. Normal involuted accessories were stimulated to a complete functional condition at any time, as were accessory organs of castrates, by injections of male hormone; their periodic low state, therefore, is not due to any incapacity to function, but to a seasonal deficiency in the supply of male hormone.

The testis has the capacity to function, during the season of low activity. Gonadotropic substances, inducing no normal stimulation of accessories in castrates, stimulate spermatogenesis and cause the testis to release enough male hormone to activate practically quiescent accessory organs to a functional breeding condition. Spermatozoon formation was accelerated in the adult and induced precociously in young males by fresh pituitaries, pregnancy urine and Antuitrin-S. Young rats, mice and monkeys have been found to differ from the ground squirrel in this respect, since such materials produced either little or no acceleration of spermatogenesis (Borst, '30; Brouha, Hinglais and Simonnet, '30; Kraus, '30;

Moore and Price, '31; Neumann, '31; Engle, '32; Molien, D'Amour and Gustavson, '33). Johnson and his associates ('31, '33, '34) have reported accelerated spermatogenesis in the adult ground squirrel, following pituitary implants. Acceleration of spermatozoon formation by gonadotropic materials, during the period of low sexual activity, has not been analyzed as thoroughly in this animal as it should be. It is possible that gametogenetic stimulation involved no more than a precocious maturation of cells, already present at the beginning of injection, with subsequent metamorphosis to form spermatids and spermatozoa; the writer hopes to give immediate attention to this question.

It is evident that the anterior pituitary is the gonad stimulating mechanism in the male ground squirrel, since pituitary implants stimulated the testis, while oestrin (reported to inhibit the pituitary of rats; Myer, Leonard, Hisaw and Martin, '30) damaged the entire reproductive system of breeding adults and inhibited sexual development in young males.

The anterior pituitary of male ground squirrels exhibits a seasonal variation in activity and, therefore, is responsible for sexual periodicity in this species. Pituitaries from males with abdominal gonads were found to have either little or no sex-stimulating capacity, while those from animals with scrotal testes had great potency; blood serum tests offered suggestive support for these results.

The testis exerts a reciprocal action on the pituitary, suppressing its activity, because castration increased the potency of pituitaries even during the season of low genital function. This reciprocal relationship, however, cannot be the only factor involved in the production of sexual periodicity, since both testis and pituitary are simultaneously practically inactive for a period of months in normal animals. Also, male hormone (in the concentrations used) failed to produce detectable damage to the testis of breeding adults, despite the fact that such treatment is followed by testicular injury in the rat (Moore and Price, '30, '32).

It is possible that male hormone (known to prolong spermatozoon viability; Moore, '27) plays a part in spermatozoon differentiation, since injections of this substance, at a time near the normal onset of accelerated sexual activity, induced a precocious formation of spermatozoa (both in adult and young males). The administration of male hormone, at a time when pituitary activity is just beginning to become apparent, may serve as an added stimulus and accelerate spermatozoon maturation. The writer plans to investigate this question with a greater degree of thoroughness.

The seasonal character of reproductive periodicity in the male of this species is convincing evidence that some environmental factor operates in producing the sexual cycle, acting either directly or indirectly on the pituitary gland. Light is probably not a major factor, despite the fact that it affects the reproductive state in some mammals and birds (Rowan, '29; Bissonnette, '31, '32; Baker and Ranson, '32; Hill and Parkes, '33). Reproductive organs undergo conspicuous development in the dark hibernation burrow, reach a peak in the spring and begin to regress at a time when there is still a progressive increase in the daily quantity of light. Progressive lengthening of the normal daily light period, prolonged addition of light and reduction of light to almost complete darkness (0.5 foot candle) failed to produce observable effects on the genital tract.

It is possible that temperature (known to condition spermatogenesis in fishes; Craig-Bennet, '31) is a factor, since low genital function is associated with a cool environment and diminished sexual activity is accompanied by hot summer temperature. Torpidity, induced by cold temperature, prolonged the time required for sexual development.

Sexual periodicity in the male ground squirrel is due to the periodic activity of the anterior pituitary gland, which must be influenced by some environmental factor. The precise controlling mechanism of the anterior pituitary remains to be determined. Studies, reported in this paper, are being continued and extended by the writer.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

(All sections cut at 4μ and magnified $\times 125$)

11 Portion of testis from a normal breeding adult, killed April 29th. Large tubules have large lumina and contain all elements of the germinal line.

12 Section of testis from a normal adult, killed on November 19th during the period of low sexual activity. Small tubules with inconspicuous lumina exhibit neither spermatids nor spermatozoa.

13 A portion of the right testis, surgically removed from a normal adult (animal number 364) on October 5th. Small tubules with inconspicuous lumina show neither spermatids nor spermatozoa.

14 A section of the left testis of animal 364. Testis removed at autopsy on November 19th after the animal had received pregnancy urine injections for 41 days. Large tubules have large lumina and contain many spermatozoa.

15 Section of autopsy testis from an uninjected 39-day unilaterally castrated young male, killed November 19th. Tubules exhibit small lumina and no spermatozoa.

16 Portion of autopsy testis of a young unilateral castrate, receiving pregnancy urine for 41 days and killed on November 19th. Large tubules have large lumina and contain many spermatozoa.

17 Section of autopsy testis from an uninjected 26-day unilaterally castrated young male, killed January 4th. Tubules exhibit inconspicuous lumina and no spermatozoa.

18 A portion of autopsy testis of a young 27-day unilateral castrate, which received 100 rat units of Antuitrin-S twice daily for 27 days and was killed on December 10th. Large tubules show large lumina and many spermatozoa.

